Is A Universal, One Dose Cholera Vaccine Possible?

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Abstract: The Gram negative bacterium, *Vibrio cholerae* (Vc) causes cholera, an enteric disease that has killed untold numbers of humans. In the 19th century, whole-cell (W-C) cholera vaccines were tested in humans. Field trials (1960s-70s) of injected, killed W-C (kW-C) Vc showed cholera-specific immune responses (antibodies, Abs) could be induced with a single dose in certain cohorts, but more durable immunity was sought with oral cholera vaccines. Newer, killed (two doses) and later modified live (one dose) W-C cholera vaccines for oral delivery were developed and extensively tested. After over 200 years, no consensus exists as to what are protective Vc antigens or how to identify them. An enduring cholera vaccine for children <5 years of age and Vc-antigen naïve individuals is still needed. The cholera outbreak in Haiti underscores some unresolved issues associated with the current cholera vaccines. Annually, is there enough cholera vaccine for those who need it? Who needs to be vaccinated and when? Given the displacement of populations during flooding like in Pakistan or in Haiti due to an earthquake, can a single dose of the kW-C cholera vaccine function to prevent or to contain an outbreak? What does 'working' entail: solely preventing deaths, or that plus long term immunity? Can a single formulation of Vc antigens be used for endemic or epidemic cholera which may require reactive vaccination for epidemics, but prophylactic vaccination for endemic cholera? The immunologic realities given the logistic issues for the different needs of a cholera vaccine are discussed.

Keywords: B cells, cholera, cholera vaccine, system biology.

INTRODUCTION

In either endemic or epidemic form, cholera is a major cause of morbidity and mortality in Africa and Asia [1]. Pathogenic Vc is difficult to isolate directly from its source-sink, nutrient-poor, brackish water [2, 3]. Vc can be planktonic (swimming cells) or biofilm-associated cells (aggregates of cells associated with other animate or inanimate objects). Vc generally exists in water as biofilm-associated cells known as conditionally viable environmental cells (CVEC) [3]. CVEC are ingested in contaminated food or water which presage seasonal outbreaks of cholera. Historically, cholera researchers have argued that identification of Vc virulence factors and their regulation should lead to a universal and hopefully one dose cholera vaccine. Much has been learned about virulence regulation in Vc [4]. The environment and host links that facilitate transmission to humans have been identified and should suggest likely cholera vaccine candidates or interdiction points [4, 5]. It has been appreciated for some time that Vc cells express protective antigens associated with their outer membrane (OM), yet new subunit or kW-C vaccines featuring OM structures have not been developed as alternatives to the oral cholera vaccines that do not (or do not optimally) express Vc protective antigens [6]. There are multiple life cycle stages of Vc during its time in the aquatic environment, and in the human host at the epithelial cell interface, that have not been exhaustively investigated for antigens able to induce durable immunity in children.

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How has cholera research prepared the world to deal with cholera outbreaks? The Pan American Health Organization (PAHO) and the World Health Organization have proposed various counter measures for Haiti's cholera outbreak. The areas that merited attention were health services, epidemiology, community prevention and social mobilization, water and sanitation, management of resettlement camps, and logistics – no mention of vaccine in the 2010-2011 report (http://bit.ly/buIbn7). The early PAHO report specifically did not recommend vaccination as a solution. Is this because the available cholera vaccines have limitations, and if so, can they be addressed?

There are many reviews on cholera vaccines and Vc pathogenesis which emphasize different aspects of the biology; the immunology, the virulence, and the ways forward to a better cholera vaccine [4-11]. It would be impossible to list all of the many excellent reviews and I apologize to those whose work is not listed – it is not a comment on the quality of their work, rather a concession to the volume of the material. The focus of this review is the integration of the immunology associated with cholera protection, the realities of protection at the gut mucosal surface given the B cells that are there, and the antigens expressed by Vc at different times during its life cycle. The overarching question is do we have all the right approaches for a one dose cholera vaccine that will work for all, at any time in their lives.

CHOLERA VACCINES AND VC IMMUNOGENS EXPRESSED IN DIFFERENT NICHES

The main limitation of the injectable kW-C cholera vaccine was its meager induction of long-term immunity, especially for young children (Table **1**). Injectable kW-C cholera

1875-0354/11

Table 1. *Vibrio cholerae* Vaccines that have been Tested in Humans

LPS (injectable)

Purified LPS [12-15]

Dose/amount of vaccine/vaccinees: One intradermal dose; Ogawa LPS (0.1-0.4 ml of 25 μg/0.1 ml, depending on age) or Inaba LPS (0.5 ml of 20 μg/0.1ml) [12-14] parenterally delivered to medical staff or Pakistani children and young adults living in endemic cholera areas.

Outcomes: No vibriocidal Ab measured for Ogawa immunized individual [13]; Inaba immunized had an average geometric mean serum vibriocidal titers some 3 months after immunization of 221 versus a baseline of 13 [13].

Efficacy of vaccine: For the study of Ogawa LPS efficacy, the majority of cholera was caused by classical Inaba, only some by El Tor Ogawa; kW-C was better at protecting than purified Ogawa LPS (year 1; 72% vs. 6%) children below 9 years of age, but Ogawa LPS was similar to kW-C for those older than 10 (kW-C; 78% vs. Ogawa LPS; 89% protection [13]. Early in the first cholera season (3 months after vaccination), Inaba LPS and Inaba kW-C vaccines were equally effective [14]. (Reasons for differences, LPS serotype, time after vaccination).

Engineered *Salmonella typhi* expressing Inaba LPS [47]

Dose/amount of vaccine/vaccinees: Three, oral doses of modified-live Salmonella given to Maryland adult volunteers.

Outcomes: Limited titers of anti-Vc LPS or vibriocidal Abs (reasons: competition with Salmonella LPS, low expression of Vc LPS).

Efficacy of vaccine: 25% protection (reduced severity of illness) after challenged with O1 El Tor Inaba.

Non-toxic LPS protein conjugates (i. m.)

Inaba; hydrazine-treated LPS conjugated to CT [17]

Dose/amount of vaccine/vaccinees: Two, parenteral dose; 6 weeks apart for (18-44 year old) 38 Walter Reed Army Institute of Research adult volunteers injected with 25 μg conjugate, no adjuvant.

Outcomes: No difference in anti-LPS IgM Ab for those immunized with Inaba-LPS-conjugate compared to immunization with kW-C ($4x10^9$ Inaba/Ogawa) Higher, anti-LPS IgG Ab in Inaba-LPS-conjugate group; better vibriocidal titers in kW-C group.

Efficacy of vaccine: no challenge.

Modified-live Vc (oral)

CVD 103-HgR (Δ A CT genetic elements, expresses CT B subunit, buffered) (no longer manufactured) [16, 88-91]

Dose/amount of vaccine/vaccinees: One dose ($\approx 5x10^{8-9}$ CFU) classical, Inaba; higher dose for those in endemic areas [90]**.** University of Maryland and University of Cincinnati volunteers immunized with CVD 103-HgR; challenged with El Tor Inaba; Immunization of adults and children (2-5 years old) living in Jakarta [89].

Outcomes: Marylanders, 91% increase in vibriocidal Ab; Jakartans had a 64-70% increase in vibriocidal Ab [89].

Efficacy of vaccine: Marylanders, 96% of vaccines were protected against severe diarrhea [16]. The first year of extensive field trial in Jakarta had a lower than normal cholera case load which influenced efficacy (14%) [89].

Killed; whole-cell Vc (oral)

Dukoral, kW-C, O1 only, recombinant CT B subunit, buffered [92, 93]

Dose/amount of vaccine/vaccinees: Three doses ($\approx 10^{11}$ CFU), 2-6 weeks apart (later trials two doses), O1 Vc, 2.5×10^{10} each: Inaba classic (heat-inactivated); Inaba El Tor (formalin-inactivated); Ogawa classical (heat-inactivated); Ogawa classical (formalin-inactivated). No O139 antigens. Bangladeshians, children (2-15) and women older than15 were immunized with Dukoral [92];

Outcomes: In a three year follow-up, protective efficacy of ≈50% independent of the B CT subunit was reported. Early after immunization (< 6 months) young children were protected as adults. Immunity rapidly waned in the young children (age 2-5). During the 5 years of follow up it was determined that protection did not extend past year 3 after vaccination [93].

Efficacy of vaccine: Protective efficacy for individuals (after 3 doses) at three years, kW-C B subunit 68% and 26% for adults and children, respectively [92].

mOCRVAC (O1/O139 *no CT subunit or buffer*) [94, 95]

Dose/amount of vaccine/vaccinees: Two, oral doses of 600 EU El Tor Inaba (formalin-killed); 300 EU of classical Ogawa (heat-killed); 300 EU classical Ogawa (formalin-killed); 300 EU classical Inaba (heat-killed); and 600 EU of O139 (formalin-killed).

Outcomes: The average geometric mean vibriocidal titers for sera obtained 14 days after second dose which were given 2 weeks apart was 497 for O1 LPS and 52.4 for O139; seroconversion was 91% for O1 and 11% for O139.

Efficacy of vaccine: No challenge; Protective efficacy in field trial all ages was 66% 8 months after vaccination [94].

Shanchol (O1/O139 no CT subunit or buffer) [96, 97]

Dose/amount of vaccine/vaccinees: Shanchol is the same formulation given with the same schedule as the Vietnamese developed mOCRVAC. Adults, 18-40 and children, 1-17 were immunized [96]. 52,000 Kolkatans age 1 or older given one or two doses [97]. 77 Kolkatans of two age groups, 1-17 years or 18-40 years were immunized once or twice [96].

Outcomes: O1 Vibriocidal titers rose >4-fold in 18-40 year olds (doses: one =65%; two =46%) and children, age 1-17, (doses: one =87%; two =82%) [96].

Efficacy of vaccine: In a large field trial (two doses), people older than one year showed a protective efficacy of 67% [97]. Same efficacy in age-groups, 1.0-4.9 years, 5.0-14.9 years, and 15 years and older [97].

Killed; whole cell Vc (injectable)

Dose/amount of vaccine/vaccinees: Meta-analysis of 16 field trials involving over a million people immunized with different formulations (kW-C or purified LPS) and strains of Vc.

Efficacy of vaccine: One dose (generally) 48% efficacy at one year (95% CI; 35 to 58%), one year of protection if less than <5.

vaccines had varying efficacy (95% CI, 35-58%) in the field [12-15]. A recent meta-analysis of injectable kW-C cholera vaccine data showed they were better than some later reports presented, and that the responses could be significantly improved with alum adjuvant, an approved, but weak human adjuvant [15]. There are three, killed oral kW-C cholera vaccines in general use today. Two include O139 antigens that complement the O1 LPS serotypes, Inaba and Ogawa antigens which are a necessary component of all cholera vaccines (Table **1**). Dukoral, a mix of classical and El Tor biotypes includes the B subunit of cholera toxin (CT) which can function as a mucosal adjuvant. The other kW-C cholera vaccines do not include extrinsic adjuvants but intrinsic components (*e.g.*, LPS, flagella, CpG DNA) which are Toll receptor agonists that have adjuvant-like qualities. CVD 103-HgR, a modified-live cholera vaccine, effectively immunized US volunteers with one dose [16]. Three newer, modified-live, oral cholera vaccines are in human trials (Table **2**). The newer modified-live vaccines are of the El Tor biotype which is the current circulating Vc strain. How the new modified-live cholera vaccines differ from CVD 103-HgR with respect to antigen profile and immunogenicity has not been demonstrated experimentally or by extensive field testing and natural challenge [9]. The oral cholera vaccines differ in the number of doses required for immunity and the responsiveness of different cohorts in the induction and time required for protective immunity and its duration. Given the different successes and conditions for priming protective Abs to prevent or moderate cholera, it is disappointing that a parallel comparison of OM structure immunogenicity for the more prominent studies has not been published. It would be of interest to know the consequence (reduced immunogenicity) to the B cell epitope of kW-C Vc immunogens that are formalin- or heat-treated or if modified live Vc express novel antigens during its gut phase that provided immunity in 8 days.

Several identified Vc virulence factors are protective antigens in animal models, but only a detoxified-Inaba LPS-CT conjugate has been tested in humans (Table **2**) [17]. Chitin binding protein A (CBP-A) has a role in chitin-based Vc colonization and induces rabbit protective Abs, but its immunogenicity in humans has not been reported [18]. LPS and TCP, both colonization factors, induce synergistic vibriocidal and protective Abs in mice [19] yet there are no reports of the immunogenicity and protective potential of extensive combinations of Vc immunogens (*e.g.*, TcpA, CBP-A, LPS, flagella, OmpU) in humans even though multivalent vaccines are in the clinic [20]. In further support of the utility of other Vc antigens, (*e.g*, cell wall antigens or cell wall associated antigens) combined with LPS as cholera vaccine immunogens are the studies from Berry's group [21-24]. They were among the first to show a non-W-C, flagella containing vaccine preparation as being a superior immunogen in mice compared to W-C Vc vaccine preparations [23, 24]. Anti-flagellar Abs (to crude flagellar fractions) inhibited access of Vc to the deep ileal crypts, thus effectively reducing the infecting inoculum [24]. The synergy between anti-flagella Abs and anti-LPS Abs, and perhaps other unidentified antigens, were noted as a possible explanation for the enhanced protective capacity of crude flagella vaccine preparations [23]. Later reports by Bishop and Camilli support that motility-related antigens are important targets for Abs that would reduce colonization [25]. From their perspective, this was mainly an anti-LPS Ab affect, but as the flagella in Vc is sheathed, anti-LPS and anti-flagella Abs would be predicted to have similar effects.

The complexities and control elements of Vc gene regulation are beginning to be appreciated and highlight the different demands of the two environments that Vc occupies (Fig. **1**) [5, 6]. Vc biofilms associate with diverse macrostructures and are important for infection of humans, transmission, and survival in the aquatic environment. Vc biofilms survive better in water than planktonic cells [26-29] and recently shed Vc biofilm bacteria are more infectious if they don't reenter the source-sink for an extended time [30, 31]. Humans that shed Vc biofilms in their stool, which subsequently enter the aquatic environment, transition in to CVEC that likely possess OM structures consistent with aquatic survival. The profile of Vc water-related antigens in source sink inocula, which humans encounter and respond to, is not known.

Whether Vc biofilm-expressed antigens (source-sink or human-host specific) would induce protective Abs that prevent or moderate infection, or disperse assembled Vc biofilms, making them more sensitive to water occupancy or immune system effectors, is not known. We don't know the extent to which protective Vc antigens track with different Vc forms (planktonic vs. biofilm), nor do we know if protective antigens vary based on Vc's most recent habitat. In aquatic niches, Vc binds chitin expressing biota in part through CBP-A, TcpA, and MSHA, which induce Abs that prevent mouse colonization [5, 18, 32, 33]. Aquatic-based Vc antigen may represent a subset of uncharacterized potentially protective antigens. The complete subset has not been identified and characterized for protective efficacy in animals let alone in humans.

VC GENE EXPRESSION DURING INFECTION – POTENTIAL VACCINE ANTIGENS?

Infant mice are used to profile Vc gene expression during gut residency. *In vivo* expression technology (IVET) uses a library of Vc cells that carry transcriptional fusions in their genome wherein activation of a proximal promoter transcribes an exogenous gene which 'marks' the area of activity. IVET has identified over 200 infection-related genes in recovered Vc cells [34]. The utility of the mouse infection model system is good as their gut environments induce 65% of the Vc transcripts isolated from Vc in cholera victims' stool. Infant mice also reproduce an important aspect of human cholera pathogenesis - the increased infectivity phenotype of gut-passaged Vc [35]. Vc transcription, measured 21 hrs after mouse infection, revealed 60% of the transcripts were important for Vc persistence in stool or the aquatic environment [29]. Ninety-three percent of Vc's ORFs (core genome) are expressed *in vitro* (nutrient-rich culture) and in infected humans (nutritional or immune stress), with 42 Vc genes (1.0%) being expressed preferentially late in infection [36]. Environmental Vc isolates share 71-88% of the core gene set with virulent O1 Vc [37]. Environmental Vc does not express ≈7% of the genes that virulent, stool-resident Vc does. While many of the unique and common Vc genes

Table 2. Experimental W-C Cholera Vaccines or Vc Vaccine Antigens Tested in Human or Animals

LPS epitopes; synthetic or purified (parenteral)

Synthetic LPS epitopes [75, 98]

Dose/amount of vaccine/vaccinees: Three doses (14 days apart) of Ogawa or Inaba synthetic hexasaccharides (10 μgs carbohydrate weight) linked to BSA; emulsified in RIBI given to mice i. p.

Outcomes: Only Ogawa conjugates induce vibriocidal and protective Ab. Both conjugate serotypes were immunogenic.

Efficacy of Vaccine: Tertiary antisera protected 100% for Ogawa conjugates; 0% for Inaba conjugates.

Detoxified LPS conjugates

Dose/amount of vaccine/vaccines: Four doses (mice 14 day interval) of hydrazine or acid-hydrolyzed Inaba LPS linked to CT.

Outcomes: vibriocidal Abs in mice [99].

Efficacy of Vaccine: Not tested

Dose/amount of vaccine/vaccines: Two dose (1 mg) for rabbits (i. m.) alkali-treated LPS (Ogawa; Inaba detoxified LPS) linked to Vc OM structures emulsified in Freund's adjuvant.

Outcomes: Three-four weeks after immunization the vaccine induced agglutinating (1:1,1024) and vibriocidal (>10^7) serum titers; Abs against LPS/OM epitopes [100].

Efficacy of Vaccine: Not tested.

Modified-live Vc (oral)

Peru 15 (ΔCT elements, added B CT gene, can be buffered) [9]

Dose/amount of vaccine/vaccinees: One dose (10^{7-9}CFU depending on study) of non-motile, non filamentous, classical Inaba given to Bangladeshi adults and children. One oral dose for 11-59 year old, US volunteers who were challenged 3 month later.

Outcomes: Induced anti-CT, LPS (vibriocidal), and TcpA Abs, the latter two were lower titer in children than older individuals; > 70% of toddlers and young children (9 months - 5 years) responded with vibriocidal Ab. US volunteers had a conversion to vibriocidal Ab of between 90-98%. Some 300 Bangladeshians were immunized with Peru 15. Children <5 seroconverted at a rate of 77% (age 9-23 months 84% conversion).

Efficacy of vaccine: Provided adult US volunteers 100% protection against severe or moderate diarrhea.

Cuba 638 (CTXPhi negative, hemagglutinin/protease-deficient) [101, 102]

Dose/amount of vaccine/vaccinees: One dose (≈10^{7-9} CFU depending on the study) of motile Peru-derivative El Tor Ogawa given to adult Cubans.

Outcomes: Induced anti-CT and bacterial Abs (vibriocidal).

Efficacy of vaccine: 100% protective against virulent Vc challenge for 12 volunteers one month after vaccination.

VA1.3 (Δ CT elements; added B CT gene, buffered) [103]

Dose/amount of vaccine/vaccinees: One dose (5 x 10^9 CFU) El Tor, Inaba given to 186 Kolkatans (16-50 years old) **Outcomes**: Induced anti-CT Abs; and vibriocidal Abs (57%).

Efficacy of vaccine: Protection not measured.

IEM108 (CTXϕ deficient, added B CT gene) [104]

Dose/amount of vaccine/vaccinees: One, intraileal dose (10^9 CFU) El Tor Ogawa to three rabbits/immunization group.

Outcomes: Induced anti-CT Abs that peaked at day 21 (average titer 1:1,455); vibriocidal serum Abs averaged ≈1:800 at 14 days post immunization.

Efficacy of vaccine: Twenty-eight days later, protection against CT or W-C challenge of immunized rabbits against fluid accumulation (ileal loops) during 16 hours test period was 100%.

Outer membrane-derived immunogens

Outer membrane vesicles (OMV) [105-107]

Dose/amount of vaccine/vaccinees: Three doses (various routes) 14 days apart of El Tor OMV; Four, oral doses of OMV; 7 days apart; 5 rabbits/group [107].

Outcomes: Both protocols induced LPS-specific vibriocidal Abs that declined rapidly; peak titers day 21 (1:100,000) [107].

Efficacy of vaccine: Three doses induced passive anti-OMV Ab immunity protected neonatal mice against oral challenge or reduced colonization of older mice [105]. Four oral doses provided 60-100% protection against oral challenge of immunized rabbits, which was strain dependent [107].

Vc ghosts [108]

Dose/amount of vaccine/vaccinees: Two oral doses (or one i. m. dose), 14 apart of El Tor, Ogawa and O139; Vc ghosts; (autolysis induced; cytoplasm loss).

Outcomes: Induced rabbit vibriocidal Abs

Efficacy of vaccine: Protective Abs against intraduodenal challenge

Proteoliposome-derived cochleates [109]

Dose/amount of vaccine/vaccinees: Two (28 days apart) or three doses (7 days apart) intranasal doses (100 μgs) of El Tor Ogawa given to mice. Cochleates are Vc calcium-extracted cells containing OM and OM-associated structures including OmpU, LPS, MSHA.

Outcomes: Three doses to achieve sIgA in saliva and feces; two doses provided IgG and vibriocidal Ab in sera.

Efficacy of vaccine: Not tested.

Table 2. contd….

Purified antigens
Dose/amount of vaccine/vaccinees: Ogawa or Inaba LPS/classical or El Tor TcpA multiple doses of both immunogens, TcpA (in adjuvant) given subcutaneous [19].
Outcomes: Not reported.
Efficacy of vaccine: 77% or 88% efficacy for classical Ogawa or Inaba and classical TcpA, respectively in the infant mouse protection assay. Anti-MSHA Ab protected against El Tor.
Dose/amount of vaccine/vaccinees: Multiple doses of TcpA peptide-KLH in Freund's adjuvant over a 56 day period given to rabbits. Quaternary sera were obtained 87 days after the first immunization.
Outcomes: No vibriocidal Ab, reduced colonization as evidenced by protection in infant mouse model.
Efficacy of vaccine: Induce 80% protection in the infant mouse model using tertiary rabbit sera, 35 days post vaccination [33].
Dose/amount of vaccine/vaccinees: A TcpF-GST fusion protein inoculated into rabbits; No details of dose, amount or number of immunization [110].
Outcomes: Induced non-agglutinating Abs.
Efficacy of vaccine: Anti-TcpF Abs increased the LD_{50} of Vc for challenged infant mice by about two logs; percent survival was not reported nor were statistics provided to determine the average log change which was likely statistically significant.
Dose/amount of vaccine/vaccinees: Rabbits Ab specific for recombinant CBP-A-His; No details of dose, amount or number of immunizations [18].
Outcomes: Not reported.
Efficacy of vaccine: Significant protection (no statistics) in infant mouse protection assay; 75% mice alive at 52 hrs post infection compared to 0% for mice given preimmune rabbit Ab.
Dose/amount of vaccine/vaccinees: Multiple doses (3-5) with semi-purified MSHA pilin in Freund's complete (first 2 doses) then Freund's incomplete given subcutaneously to rabbits [19, 111].
Outcomes: 76% efficacy against El Tor MSHA; also effective in ileal loop fluid accumulation assay.
Efficacy of vaccine: Induced protective Abs for infant mouse assay; percent not reported.
Dose/amount of vaccine/vaccinees: Rabbits received gel purified OmpU in Freund's complete adjuvant for first dose; the final two doses were in Freund's incomplete.
Outcomes: Prevented adhesion to epithelial (*in vitro*).
Efficacy of vaccine: Induced protective Abs for 100% of the 5 mice/group used for infant mouse assay [112]
Dose/amount of vaccine/vaccinees: Vc El Tor flagella antigens present in isolated OMs were used to immunize rabbits
Outcomes: A 33 kDa flagellar antigen inhibited Vc mouse colonization and reduced virulence in the rabbit ileal loop model [113-114].Abs specific for a 40 and 38 kDa flagellar antigen inhibited motility; but not virulence in rabbit ileal loops. Flagellar [21-24] or Anti-LPS Abs inhibited flagella-based mobility due to the LPS-sheathed flagella [25].
Efficacy of vaccine: Induced protective Abs for infant mouse assay [25].

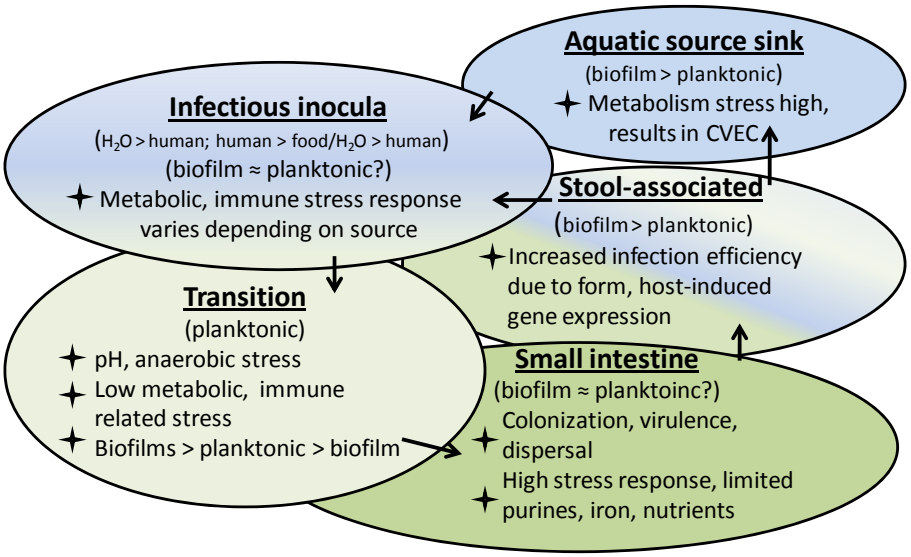

Fig. (1). ***Vibrio cholera's*** **life cycle is complex with respect to gene expression in different niches.** Water is the natural source-sink for Vc (O1/O139) and hundreds of other *Vibrio* species. Environmental and social conditions can enhance the entry of Vc into susceptible humans who can become infected directly from the source-sink or from fecal contamination of household food and water. The transition from water or stool to manifestation of cholera is different with recently stool-shed Vc contaminating water or food being more infectious than source-sink Vc due to a phenotype fashioned by gut residence and passage. The biofilm form of Vc is important for survival and infectivity; but the antigen profile of this population and the transcriptome needs to be established to evaluate the potential of controlling or moderating Vc biofilms (entering or exiting humans) as part of the protective process.

expressed are internal antigens, those that are not need to be evaluated relative to vaccine targets that could function at different points in Vc's life cycle.

For some of the unique Vc OM structures expressed at the human epithelial barrier, it is unclear whether they are protective antigens, as a significant number are either listed as unknown or hypothetical [34]. The 'demands' on Vc during infection and for egress from humans include nutrient (e.g. iron) acquisition and motility, but not chemotaxis [36, 38]. Vc proteins isolated from cholera patients' stool confirmed skewing of gene expression for protein synthesis and energy metabolism [39]. High levels of transcripts for OmpU and multiple OM structures (OmpS, OmpV, OmpK, OmpC, OmpW, and OmpA) were present along with a number of conserved hypothetical proteins. An *ompU* (a general porin) prologue, *vca1008*, identified by IVET is required for mouse colonization [40]. Subunit configurations of the porins (in liposomes) or over expression of them for kW-C vaccines has not been extensively reported as an approach for improved cholera vaccines. A reasonable hypothesis is that OM structure-specific Abs binding their target antigen would increase metabolic stress by blocking nutrient acquisition or interrupting function, thus increasing the energy demands on Vc to a tipping point to cause gene activation events consistent with relieving the stress in the human host by redistributing back to the environment [41].

Vc's proteome has been published, but it has not been linked to the human immunome (available Ab specificities), nor has it been tested against a comprehensive panel of Vc convalescent sera or sera of those who are hyperimmune to cholera [42]. While the *in vivo* expressed OM proteome is a place to start the immunome analysis, the presence of Vc-specific Abs in cholera patients does not prove a role in protection. Similarly, the lack of Abs does not indicate that there is not a role for that immunogen in protection following vaccination with the immunogen. Even though natural, acute Vc infections induce better immunity (> 3 years) than cholera vaccines, natural infection or vaccination may never 'present' all of the potentially protective Vc immunogens. Thus, inclusive methods must be used to understand what Vc OM-structures or associated structures could be protective immunogens.

Antibodies that bound PilA, TcpA, and OM-associated structures, PilQ, MSHO, MSHP, and CapK were found in cholera convalescent serum by *in vivo*-induced antigen technology [43]. The test sera were extensively adsorbed to discover Abs to antigens *not* associated with *in vitro* grown Vc or internal antigens. The immunogenicity of components of MSHA (MSHO, MSHP), TcpA, and PilA (PilA, PilQ) were variable. This study highlighted an issue of importance for cholera vaccine development: what is the limit of valid comparisons of mice and human anti-Vc antigen. The three pili are immunogenic for both humans and mice. TcpA is required for human and mouse colonization, MSHA and PilA are not required for colonizing mice [44-46]. Immunogenicity of macrostructures is likely transferable between species, but protective efficacy which relates to the biology of the infection is likely system dependent. Thus the protective capacity of anti-OM structure Abs ultimately needs to be assessed in humans. This is clearly a rate limiting step in cholera vaccine development.

A ONE DOSE CHOLERA VACCINE: EVIDENCE, SCOPE, STRATEGIES, AND LIMITATIONS

The parenteral kW-C cholera vaccines, CVD 103-HgR (excellent vaccine for USA-based volunteers) and *Salmonella typhi* expressing Vc Inaba LPS induced variable protection for humans with one dose [12-15, 47]. Given the historic caveats about cholera immunity in different populations, the potential for a one dose cholera vaccine may need to be parsed relative to whether the vaccine is intended to prime or boost Vc immunity in a specific population. The time required for this is also an important parameter. Other than BCG, most bacterial antigen-based vaccines require multiple doses for optimal immunity [48]. Thus a critical question: is a one dose cholera vaccine, that induces durable protective immunity in young children possible or, are two doses with routine boosters the only way to induce and maintain immunity in the age group 2-5 years old?

If age and immunologic experience with Vc antigens are an intractable part of the immunologic metric for mucosal priming by a one dose cholera vaccine (using the current oral cholera vaccines), it may be difficult to realize a universal vaccine without extensive redesign based on what Vc antigens can induce germ line protective Abs, which will be required for the young and impoverished or those completely naïve to Vc antigens. We need to determine if the Vc antigen matrix can mitigate the protection metric. Can kW-C vaccine be redesigned to enhance certain OM structure expression or to provide routine inclusion of biological response modifiers or adjuvants? We need to determine if Vc OM antigens delivered orally can be complemented by parenterally induced IgG and IgM specific for the same Vc OM profile or for another set of structures which intrinsically do not prime gut mucosal response, but do prime peripheral (*e.g.*, spleen) or nasal mucosal immune responses, both of which can supplement the gut mucosal immune response of young or Vc-antigen naïve individuals. Can intranasal immunization, which requires less vaccine antigen and involves less intrinsic antigen competition, be used to supplement oral kW-C vaccination? Which OM associated Vc antigens should be pursued? Current kW-C cholera vaccines do not express, or present to the immune system, all possible Vc protective antigens especially those OM-structures associated with gene activation at the epithelia or secreted proteins.

The kW-C cholera vaccine stimulates good immunity in people living in endemic cholera areas, proving protective Vc OM immunogens exist. These responses may be anamnestic responses to dominant Vc antigens which poorly prime the Vc-naïve or the immune systems of young children. We don't know the complete profile of Vc protective immunogens, nor if all are equally immunogenic for different aged individuals. We don't know if kW-C and modified-live vaccines induce the same Ab responses, or if priming to LPS or OM antigens differs based on whether the Vc cells are killed or viable. This makes it difficult to determine what is limiting for those who do not respond well to the current oral W-C cholera vaccines. Anti-LPS Abs predominate following Vc immunization, underlining the need to know how to broaden the response to other protective OM immunogens. Knowing the complete W-C OM profile, and how the OM structures vary with Vc life cycle stages is a first step

to engineering a better cholera vaccine, whether a kW-C, modified-live, or subunit vaccine.

Recombinant antibody, phage libraries and cloned Abs from EBV immortalized human B cells are a proven technology and a direct means of defining Vc OM structures [49]. Comprehensive OM-Vc specific Fab (antigen binding fraction, immunoglobulin variable domains of the heavy and light chains) isolation is possible due to the tremendous, preformed diversity of recombinant Ab libraries, which eliminates the difficulties of inducing Ab *in vivo*. Fab-identified, Vc OM structures should be ranked for immunogenicity and protective potential after a single dose in mice or rabbits. We should search for protective OM structure specific Ab that *do not* require *any*, or *extensive* somatic mutation in their antigen binding sites, as these germ line configured Abs would be expected to be induced with one dose of immunogen. The search for protective antigens should not exclude extracellular Vc components like CBP-A, which provoke titers of over 1:1,000,000 in 21 days in mice with single immunization (WF Wade, personal observation). Other OM-specific Abs that do not directly affect colonization or virulence factor function, but nonetheless increase the 'stress' on Vc, should be identified [41]. OM structures associated with nutrient acquisition (porins), iron transporter, and antimicrobial peptide defense are likely targets for Abs which would supplement more traditional anti-colonization or virulence factor specific Abs.

ACTIVATION OF MUCOSAL B CELLS, B CELL SUBSETS, AND B CELL MEMORY

Vc's only natural vertebrate host is humans, but mice and rabbits are used to study Vc pathogenesis and immune responses to Vc antigens. Differences in pathogenesis, gut flora, Ab repertoire diversification, and B cell subsets suggest prudence in directly interpreting vaccine related results obtained with non-human species [50]. The gut is a major production site of natural Ab, low affinity cross reactive Ab that controls endogenous flora, as well as pathogen-specific Abs that protect the host [51-53]. Antigen, along with age-related factors, influences competition among different specificities of mucosal B cells. Depending on the type of B cell involved, B cells develop into memory B cells or long term plasma cells (Ab producers). The latter require survival niches to manage steady state Ab production, which in conjunction with memory B cells provide long term humoral immune protection [54]. Dendritic cells (DC) and lamina propria (LP) macrophages are important for mucosal Ab production as they direct antigen transport and presentation [55, 56]. In response to TLR agonists (*e.g.*, LPS, flagella both expressed by Vc) these cells produce cytokines such as BAFF (B cell activation factor) and APRIL (A proliferation induced ligand), which along with IL-6, and IL-5 support B cell viability, switching to IgA, and differentiation of plasmablasts (short term Ab producers) and plasma cells [51, 57-59]. In mice, an LP DC subset responds to flagella by secreting multiple factors required for extrafollicular IgA production [60]. The role of this DC subset in cholera immunity is unknown.

Human B cell subsets are differentiated based on surface phenotype, function, and location. B cell subsets can have different activation requirements, different potential for somatic mutation, different propensities to enter the memory cell pool, and somewhat specialized Ab repertoires [61-66]. Follicular and marginal zone (MZ) B cells are B cell types found in Peyer's patches' (PP) germinal centers (GC). Follicular B cells also are found in isolated lymphoid follicles that can play a role in immunity [66-70]. The human B cell equivalent to mouse B1 B cells that secrete 'innate or natural" IgA have not been conclusively identified, but humans do make 'innate' Abs [66, 71]. A source of human 'innate' Abs (*e.g.*, anti-carbohydrate, anti-LPS) is MZ B cells and in mice B1 B cells. Marginal zone B cells can be located in the subepithelial dome above PPs [64]. Circulating, $CD27^+$ MZ-like B cells that independently of antigen, acquire somatically mutated Abs are present by one year, but can take 10-15 years to attain the adult MZ-like B cell repertoire [71, 72]. An important consideration for cholera vaccine development is identification of the B cell subsets that respond best to Vc antigens, in particular LPS as MZ and B1 B cells are known to respond well to T independent antigens like LPS.

Memory B cells are thought to mainly derive from GC reactions, suggesting Vc antigens have to travel to the PP or draining lymph nodes and receive antigen-specific T cell help to induce B cell memory [62]. The molecular machinery for somatic mutation is found in GC and in some extrafollicular B cells. The extent of the somatically diversified, human anti-Vc LPS Ab response is not known [51, 67, 68]. Blood-borne B cells, 7 days after cholera infection are thought to represent gut-activated B cells ($CD27^+$) in transit back to the LP [73, 74]. The presence of other CD27+ B cell subsets, some of which are not recently activated is a confounding factor to the identification of recently activated cells or memory cells. Kinetic analysis showed an initial short-lived, anti-Vc LPS (T-independent) B cell response and a subsequent longer-lived CT (T-dependent) response. CT induces GC reactions and subsequently higher affinity Abs. We don't know if young children respond to these antigens with the same kinetics and magnitude, but if so, that would indicate equivalent gut priming of the B cell response and that plasma cell survival niches may be limited in the young and thus explain the shorter duration of immunity. In mice anti-Vc LPS Abs have characteristics of mutated sequences [75, 76]. Mice have a population of splenic MZ B cells that respond very fast and robustly to LPS [77]. It is important to know the source of human Vc LPS-specific Abs and determine if and where somatic mutations occur and whether they are limited by age as MZ-like B cells are [61]. This may suggest a very specific population of B cells to target Vc antigens like LPS to.

Oral cholera vaccines were designed to directly stimulate mucosal immunity and eliminate inoculation site complications. Solving one problem uncovered another, priming mucosal immunity which is very dose dependent and conditions–based, due to antigen competition and the intrinsic issues of immunologic tolerance in the gut [78]. Priming murine gut-associated B cells is different than peripheral priming to the same antigen [78]. Mucosal responses are slower to accrue and boosters are additive not synergistic as with parenteral immunization [79]. The different rules for gut Ab production and instillation of mucosal memory may preclude oral cholera vaccines from ever being a universal,

one dose, long term vaccine. Perhaps modifications of immunization protocols or the vaccine antigens can be used to 'overcome the rules of the gut' and induce immunity with one dose of vaccine. If not, this does not mean that the efficacy of a two dose vaccine schedule cannot be improved to broaden and lengthen immunity to the maximum obtainable given the 'rules of gut immunity'. One modification to contemplate is enhanced expression of selected protective antigens from specific life cycle stages. Targeting Vc antigens to GCs (*via* microfold antigen acquisition cells in the gut) by delivery vehicles to avoid competition for access to PPs' GC, and biological response modifiers to maximally 'activate' the immune system should be evaluated as well.

ISSUES IN ACADEMIC AND REAL-WORLD VACCINE DEVELOPMENT – DO THEY COINCIDE

The presentation of targetable, protective antigens that the available Ab repertoire can respond to is central to Ab-mediated immunity. Some data suggest there is only one proven protective Vc immunogen: LPS [12-15]. It is likely that there are numerous Vc protective antigens past LPS. Does cholera immunity always involve steps to prevent colonization? Maybe we need to think more broadly about how to reduce or mitigate cholera in the young in addition to reducing or preventing colonization. Is there a vaccine role for antigens expressed only at the Vc: mucosa interfaces or later at dispersal that would reduce the virulence of stool associated Vc biofilms? How many different Ab targets are expressed by aquatic associated Vc that could be used to influence its virulence or prevent its ability to cause disease for the few Vc colonizing pioneers that might elude anti-colonization Abs and the first wave of innate immunity? Often in experimental cholera vaccine research, new vaccine immunogens are not tested with different protective mechanisms in mind or by one dose immunization protocols which are often cited as an important component of a future cholera vaccine (Table **2**). We know that some Vc colonization and adhesion factors induce protective Abs, but they are not featured components of kW-C cholera vaccine even though they might greatly supplement the anti-LPS Ab response. If cholera immunity requires 10,000 Abs to a particular structure, which requires multiple immunization to achieve, but individual antigens (*e.g.*, LPS, TcpA, CBP-A) directed at a common step in pathogenesis can induce 2500 Abs with one dose, then one vaccination with four such immunogens will be protective. This concept does not include the additive or synergist role Abs against OM targets that would affect pathogenesis steps downstream of colonization. The technology exists to enrich for and pick the best age-appropriate Vc immunogens from the different antigen sets Vc expresses.

The recent flooding in Pakistan and Bangladesh drew attention to unresolved issues of cholera vaccine delivery and compliance if two doses of an oral kW-C vaccine are absolutely required to induce cholera immunity in a developing epidemic setting [80]. In epidemic cholera the transmission mode is amplified due to the naïve Vc-antigen status of the people, and typically a large percentage of people need successful vaccination in a short time to effectively break transmission, a circumstance that is less likely for the immediate outbreak area especially if it is large [81, 82]. A recent report by Chao and coworkers discussed targeting one million doses of killed, oral cholera vaccine to 5% of those in areas with high exposure to Vc predicting that it would reduce the number of cases by 11%. If 30% of the population were targeted with attending modest hygienic improvement, they model that the cases of cholera would be reduced by 55% and 3,320 deaths prevented [83]. The one dose Peru-15 cholera vaccine or its equivalent may overcome some logistic issues (vaccine distribution, immunity in a short time based on two doses with the killed cholera vaccine) for epidemic cholera. Will Peru-15 and its analogs also immunize young children to reduce disease in endemic areas or prevent epidemic cholera in young children? Some suggest vaccination of the transmitters of cholera could be very effective. This does not seem to be the case in endemic cholera as many are immune, but the susceptible cohort still persists.

Do these circumstances of cholera infection suggest different cholera vaccines for different situations based on age, location, and the speed with which immunity must be induced? A recent review by Dr. Levine clearly documents the 'issues' associated with the efficacy of oral vaccines in developing countries [8]. This is due to multiple parameters which include the nutritional and immune status of the vaccinees, the latter of which is influenced by nutritional factors and concurrent infections. Perhaps we do have an effective cholera vaccine (one or two doses) for 'most' of the adults in the world especially if they live in endemic areas. If so, development focus should be determining what is needed to supplement the existing cholera vaccines for the intrinsically susceptible cohorts, independent of whether the improved vaccine configuration enhances immunity of those already responsive to existing cholera vaccines.

Vaccine development can take 10-20 years and billions of dollars. It's time to make decisions about unresolved questions in cholera vaccine research to determine the commitments that can or should be made: 1) What will we pay to develop and use a one dose universal cholera vaccine?, 2) What can be done within the current cholera vaccine framework to improve their efficacy for the young if we find better age-appropriate protective antigens?, 3) Given the success of the oral cholera vaccines for adults, especially those living in endemic cholera areas, will we support future research based on a systems approach to find Vc antigens and immunization protocols that will effectively work for young children and those naïve to Vc antigen?, and 4) If current oral cholera vaccines are not able to incorporate the 'best' antigens for the susceptible, will we use the results of basic research Vc and mucosal immunology to build a 'sure shot' designer subunit vaccine to protect the most susceptible, even if such a vaccine must be injected more than once and costs more than kW-C cholera vaccine?

WAYS FORWARD

The limitations of the mucosal immune system and the protective capacity of the peripheral immune system of young children and Vc-antigen naïve adults that have poor nutrition and co-infections needs to be integrated into cholera vaccine research. The Vc research community needs new immunologic tools to develop an improved (immunity with one dose, universal coverage) new cholera vaccine or to improve the existing cholera vaccines. An extensive panel of Vc OM-specific, phage-derived probes can readily be iso-

lated and tested for protective efficacy is an easily assessable tool [49]. OM-specific reagents would come from human recombinant Ab phage, (naïve or immune) Fab libraries. Naïve libraries exist. Cholera-immune libraries should be made from mucosal B cells of young children and adults living in endemic cholera areas.

My laboratory has constructed Vc indicator cell lines that express Vc stress- or virulence-related promoters that drive fluorochromes (*e.g.*, GFP) expression for *in vivo* analysis of how OM-specific Abs regulate Vc biology. Our Vc-promoter constructs are similar in concept to that described by Dr. Schoolnik's group that used the *tcpa* promoter to drive GFP expression [84]. Gene expression analysis of Vc indicator cells expressing different Vc promoter-fluorochromes, isolated directly from the gut by flow cytometric-based sorting, will not be limited like IVET or Vc microarray analyses that feature incomplete Vc genome coverage, unknown expression points during transit, sample averaging, and reduced sensitivity due to contaminating mRNA. Genomic approaches to comprehensively define vaccine antigens have greatly expanded the number of antigens that are isolated, tested, and moved into clinical trials [85]. Vc indicator cell lines isolated from the small intestines that reflect Vc cells expressing a particular 'global' phenotype would allow precise definition of Vc gene expression, and provide a screen to determine which OM- or OM-associated structures bound by Abs regulate Vc biology.

The Vc research community has not developed immunologic reagents such as Vc antigen-specific B cell receptor (BCR) or T cell receptor (TCR) transgenic (Tg) mice that could be used to identify the critical steps needed for induction and maintenance of mucosal cholera immunity. BCR Tgs can be expressed in different B cell subsets and keep intact memory B cell and plasma cell differentiation [86]. My group is constructing Vc Inaba LPS, BCR Tg mice using the heavy chain 'knock-in' strategy that allows some upstream heavy chain variable gene rearrangement to the knocked in gene that functionally expands the LPS-specific BCR Tg mouse to a Vc 'antigen' BCR Tg mouse [86]. Vc LPS-specific BCR Tg bred to TCR Tg mice reactive with ovalbumin would be informative to determine how detoxified-Vc LPS-ovalbumin conjugates or ovalbumin-expressing Vc prime oral anti-Vc LPS Ab responses or Vc-associated T cell responses. Intravital microscopy is used to visualize, in real-time, the *in situ* movement of DC and antigen-specific B or T cells in response to antigen that access the gut or its underlying mucosa [87]. Mice infected with the Vc indicator cell lines would show where (subepithelial marginal zone or PP's follicular) and when fluorochrome-labeled DC acquire Vc antigens for presentation to Vc LPS-B cells. The limiting interaction steps in immune system induction for different forms and OM composition of the current cholera vaccines with different aged mice can be identified with this systems approach. We will finally be able to see a gut immune response to cholera in all its detail. This knowledge should provide abundant information for translation to the clinic and the field.

CONCLUSION

If *currently* configured oral cholera vaccines and the immunologic limitations of young children and Vc-antigen naïve populations prevent universal one dose cholera vaccine coverage, do we stop there or find an antigen set and immunization approach capable of universal coverage, or do we stay with the two dose schedules that work within a large percentage of the population? Can we build a better cholera vaccine with what we now know about Vc pathogenesis and virulence? Proven protective antigens like TcpA (identified in 1987), TcpF, and CBP-A have <u>not</u> been tested in limited phase 1 trials. Why bother identifying Vc virulence factors as vaccine candidates if their safety, immunogenicity, and efficacy are not pursued in human trials? Should hypothesized costs based on unknown delivery modalities be the major consideration for developing a 'new' cholera vaccine? If not, then what factors should be included in the calculations?

The magnitude and costs of the contemplated studies, if all potential OM Vc antigens are tested in humans, seems daunting. A closer look suggests this is NOT a *prima fascia* argument against a systems approach in mice that would marry basic Vc and mucosal vaccine research to developing better enteric vaccines in general and a cholera vaccine in specific. A cholera vaccine systems model would generate novel data relatively quickly that might guide the development of a one dose, human cholera vaccine for all who need it. A one dose cholera vaccine may cost more than the current W-C cholera vaccines but costs could be supplemented by philanthropic organization, redirected research money, and decreased human costs due to improved duration of cholera immunity. This cost calculus needs to include the value of an effective formulation, based on a goal of >70% protection in all vaccinees for 3-4 years, that with the attending herd protective affects would be highly successful (>90%) at preventing cholera transmission or infection in setting of endemic and perhaps epidemic cholera. The way to search for *a* 'magic' cholera bullet should be reshaped to find *all* the right 'cholera bullets' and then to deliver them in the correct vaccine configuration for a universal solution to the age old scourge. If the principal policy-makers agree that a systems-based cholera research program is approachable and valuable, then derivatives of that system will advance our practical knowledge of how to 'pick the immunogen winners' for particular immune system states (the young, the malnourished or both). Better vaccines will follow that could change the paradigm of what we can afford for cholera vaccines.

ABBREVIATIONS

APRIL	=	A proliferation induced ligand
Ab	=	Antibody
Abs	=	Antibodies
BAFF	=	B cell activating factor
BCG	=	Bacillus Calmette Guérin
BCR	=	B cell receptor
CapK	=	Capsular antigen K
CBP-A	=	Chitin binding protein
CT	=	Cholera toxin
CVEC	=	Conditionally viable environmental cells
DC	=	Dendritic cell

Fab	=	Fraction antigen binding
GC	=	Germinal centers
GFP	=	Green fluorescent protein
IgG, IgM	=	Immunoglobulin [isotype]
IVET	=	*In vivo* expression technology
CpG	=	Hypomethylated cytosine and guanidine
CI	=	Confidence interval
CVD	=	Center for Vaccine Development
kW-C	=	Killed whole-cell
OM	=	Outer membrane
LP	=	Lamina propria
LPS	=	Lipopolysaccaride
MSHA	=	Mannose sensitive hemagglutinin [letter for protein of the operon]
MSHP	=	MSHO
MZ	=	Marginal Zone
HgR	=	Mercury resistance
ORF	=	Open reading frame
OmpA, C,K, S, U, V, W	= =	Outer membrane protein (letter to designate protein)
PAHO	=	Pan American Health Organization
PP	=	Peyer's patches
PilA; Q	=	Pilin [letter for protein]
TCR	=	T cell receptor
TCP	=	Toxin coregulate pilus
TcpA, TcpF	=	Toxin co-regulated pilus protein (letter to define protein)
Tg	=	Transgenic
W-C	=	Whole-cell
Vc	=	*Vibrio cholerae*

CONFLICT OF INTEREST

I have no conflicts of interests.

ACKNOWLEDGEMENTS

I thank Drs. R. Hall (NIH, NIAID), P. Velázquez (Indiana University School of Medicine - South Bend), W. Hickey (Dartmouth Medical School) and, C. Grandjean, Université de Picardie Jules Verne for critical reading of the manuscript, useful comments, and helpful discussions

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Received: May 16, 2011 Revised: June 01, 2011 Accepted: September 14, 2011